

**RESEARCH ARTICLE**

# The Effect of Propolis Extract on MDA Levels (*Malondialdehyde*) and Sperm Quality on Epididimidis

## (*Experimental Study on Wistar Strain Male Rats Exposed to Kretek Cigarettes*)

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**ABSTRAK**

**Pendahuluan:** Paparan asap rokok dapat meningkatkan radikal bebas dan berpengaruh terhadap infertilitas pria. Propolis sering dipakai sebagai suplemen untuk meningkatkan kadar antioksidan.

**Tujuan:** membuktikan pengaruh ekstrak propolis terhadap kadar MDA dan kualitas spermatozoa pada epididimidis akibat paparan asap rokok.

**Metode:** Penelitian eksperimental post test only control group design menggunakan 30 ekor tikus jantan Galur Wistar, berusia 8 minggu, berat badan 200 – 250 gram, dikelompokkan menjadi 5 kelompok secara random. K-1 (normal), tidak mendapat propolis dan asap rokok; K-2 (kontrol negatif) diberi paparan asap rokok 4 batang/hari, tidak mendapat propolis; K-3, K-4, dan K-5, selain mendapat paparan asap rokok 4 batang/hari, masing-masing diberi propolis dengan dosis 2.9 mg, 5.4 mg, dan 8.3 mg/200g BB/hr selama 21 hari. Hari ke 22 tikus dimatikan, sperma dikeluarkan dari cauda epididimidis, kemudian diperiksa kadar MDA dan kualitas sperma. Analisis statistik yang digunakan adalah one way ANOVA, dilanjutkan post hoc LSD dengan tingkat signifikansi  $p < 0,05$ .

**Hasil:** Uji ANOVA terhadap kadar MDA dan kualitas spermatozoa diantara kelompok menunjukkan perbedaan yang bermakna,  $p = 0.005$ . Uji post hoc menunjukkan bahwa kadar MDA pada K-5 (1,12) lebih rendah secara bermakna dibanding K-2 (5,89),  $p = 0,001$ . Demikian pula dengan konsentrasi, motilitas, dan morfologi (kualitas spermatozoa) yang menunjukkan bahwa K-5 (49.32, 64.15, 36.34) lebih tinggi secara bermakna dibandingkan K-2 (22.62, 27.27, 21.39), masing-masing dengan  $p = 0.001$ .

**Kesimpulan:** pemberian ekstrak propolis 8,3 mg/hr selama 21 hari terbukti meningkatkan kualitas spermatozoa dan menurunkan kadar MDA pada epididimidis tikus jantan galur Wistar yang dipapar asap rokok.

**Kata kunci:** Asap rokok, Ekstrak propolis, kualitas spermatozoa, kadar MDA

**ABSTRACT**

**Introduction:** Cigarette smoke exposure increase free radicals and effecting male infertility. Propolis is commonly used as supplement to increase antioxidant levels.

**Objectives:** To prove the effect of propolis extract to MDA level and spermatozoa quality on epididymis due to cigarette smokes.

**Methods:** Experimental research post test only control group design using 30 male rats Wistar Strain, 8 weeks old, weighed 200 – 250 gram, randomly divided into 5 groups. K-1 (normal), do not received propolis and cigarettes smoke; K-2 (negative control) exposed to cigarettes smokes 4 buds/ day, did not received propolis; K-3, K-4, and K-5, apart from cigarette smokes 4 buds/day each were given propolis with 2.9 mg, 5.4 mg, dan 8.3 mg/200g body weight/day for 21 days. On the 22nd day, rats were sacrificed, sperms were extracted from cauda epididymis, and then examined for MDA and sperm quality. Statistical analysis used was one way ANOVA, continued with post hoc LSD with significance level of  $p < 0,05$ .

**Results:** ANOVA test on MDA level and sperm quality between groups showed significant difference of,  $p = 0.005$ . Post hoc test indicated that MDA on K-5 (1,12) is significantly lower compare to K-2 (5,89),  $p = 0,001$ . As well as concentration, motility and morphology (spermatozoa quality) which indicated that K-5 (49.32, 64.15, 36.34) is significantly higher compared to K-2 (22.62, 27.27, 21.39), each with  $p$  value of  $p = 0.001$ .

**Conclusion:** extract propolis 8,3 mg/day for 21 days is proven to increase spermatozoa quality and lower the MDA level on male rats wistar strains' epididimidis exposed to cigarettes smoke.

**Keywords:** Cigarette smokes, Propolis extracts, spermatozoa quality, MDA level

**INTRODUCTION**

Smoking increase the Reactive Oxygen Species (ROS) levels, decrease antioxidant levels in semen, creating DNA cellular fragmentation and

spermatozoa's morphology abnormalities (head, neck and tail) (Sukmaningsih A, 2009). These were proven by the increasement of MDA level and 8-OhdG (DNA fragmentation marker) of 50% on male smokers'

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spermatozoa (Quaratul'ainy S, 2006). Smoking is not only harming the active smokers, but also the surrounding individuals or non smokers/ passive smokers (Rahmatullah P, 2009). Concentration or numbers of spermatozoa on active smokers are 23% lower compared non smokers (Aina N, 2005). Some studies about chemical effects of cigarettes indicated that there is disorders on spermatogenesis through increasing of free radicals production (Agarwal A, *et al.*, 2003).

ROS in higher concentration can cause lipid peroxidation and effecting on cell death (Agarwal A, *et al.*, 2005). Beside damaging cell structures, ROS also damaging DNA molecules through breaking of single and double DNA, nucleocid deletion and modification (Subash P, *et al.*, 2010). The presence of free radicals can cause spermatozoa disturbances as much as 30% to 80% from infertile male cases (Tremellen K, 2008). Infertile male are often have low antioxidant capacity on seminal plasma and spermatozoa, causing the spermatozoa prone to oxidative stress (Tunc O, 2009). Antioxidant acts as free radicals scavengers to protect spermatozoa from ROS effect.

Propolis extracts which contains Caffeic acid phenethyl ester (CAPE), a flavonoid which is good as antioxidant (Gheldof, N, *et al.*, 2002), have better antioxidant activities to fight oxidant and free radicals ( $H_2O_2$ ,  $O\cdot$ ,  $OH\cdot$ ) compared to other bee products (Nakajima, Y, *et al.*, 2009). Therefore, the use of propolis extract can prevent the free radicals and oxidative stress (Mot, A.C, *et al.*, 2009), are rational measures. Propolis extract which is beekeeping product contain CAPE can protect sperm from DNA damage caused by benzopyrene reaction with reactive oxygen species exogen (Russo A, *et al.*, 2006). Antioxidant substances are needed to neutralize free radicals and prevent damages caused by free radicals on normal cells, proteins and fats. Various studies indicated that smoking cause the decrease of quality parameters (amounts, morphology and motility) of spermatozoa (Nadeem F, *et al.*, 2012, Zhang ZH, *et al.*, 2013, Somwanshi SD, *et al.*, 2012). Other research also evidenced that cigarettes caused damage in sperm DNA marked by increased level of MDA and 8-OHdG as indicator of damaged DNA as the effect of oxidative stress (Agarwal A, *et al.*, 2014). CAPE contained in propolis is one of the strongest antioxidant substance (Farooqui, T, *et al.*, 2010; Koyu A, *et al.*, 2009, Hlavackova L, *et al.*, 2009), proven to inhibit formation of lipid peroxidation of 75.3% (Gocer H, *et al.*, 2011). The difference was because CAPE is categorized in phenolic acid hydroxycinnamic contains  $CH_2 = CH-COOH$ , so it has high capacity of electron

donor (Gocer H, *et al.*, 2011). Which means, have very high activity as antioxidant. Referring to the process of antioxidant, the CAPE contained propolis the most rational choice as natural antioxidant. The result of in vitro on ejaculates indicated that propolis is proven to fix *mitochondrial respiratory* efficiency, therefore improving the spermatozoa motility (Cedikova M, *et al.*, 2014). Other research on wistar strain male rats, the treatment of propolis dose 3 mg, 6 mg, and 10 mg/kg bodyweight/day for 56 days, is proven can increase the spermatozoa production, height of epiteliu epididimis, and no increase of ROS (Capucho C, *et al.*, 2012). The objective of this study is to prove that propolis extract can fix the decrease of sperm quality and MDA levels due to cigarettes' smoke exposure.

## METHODS

This research is an experimental research with *Post Test Only Control Group Design*. The numbers of 30 male rats wistar strain, aged 8 weeks, weighed 200-250 grams were randomly grouped into 5 groups each contains 6 rats. Normal group (K-1) do not received propolis and cigarettes smoke. Negative control group (K-2) do not received propolis and exposed to 4 cigarettes buds smoke/ day and propolis extract with dose 2.9 mg/200 gram body weight, 5.4 mg/200 gram body weight and 8.3 mg/200 gram body weight for 21 days. Those propolis extracts doses were converted from recommended adult human dose as much as 3 x 100 mg, it means 300 mg/day, while converted doses on rats with body weight BB 200 gram is 0.018. Furthermore, the converted doses for 200grams rats is  $0,018 \times 300 = 5.4$  mg/200 gram Body weight/day. Food and water were provided by adlibitum for all groups. Rats were terminated on day 22, and then examined for the MDA and quality of spermatozoa (amounts, morphology, motility) according to WHO standard 2010 edition. While the MDA levels were measured quantitatively using Thiobarbituric acid reactive substance (TBARS) assay method (Sherwood L, 2011). Methods used were TBARS with fluorophotometric and conducted on rats cauda epididimic organ. Principals of the TBARS analysis are lipid hydrolysis peroxidation with heatings, so the attached MDA will released and reacted with TBA in acidic to form red MDA TBA and measure in wave length 532nm. Statistical analysis used were one way ANOVA, continued with *post hoc* LSD with significance level of  $P < 0,05$ . This research was conducted after receiving credentials from Faculty of Medicine Unissula Ethical Committee No.013/I/2016/ komisi Bioetik.

## RESULTS

After 4 cigarettes buds exposure per day and propolis extract administration for 21 days, the results are illustrated on Table 1.

### 1. MDA Levels

Referring to table 1, the highest MDA level mean is in group K-2 ( $5.89 \pm 0.50$ ), followed by K-3 ( $3.22 \pm 0.20$ ), group K-4 ( $1.81 \pm 0.10$ ), K-1 ( $1.24 \pm 0.13$ ), and the lowest is in group K-5 ( $1.12 \pm 0.10$ ).

The ANOVA result indicated that the MDA levels mean between groups showed significant difference of,

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$p = 0.01$ . The Post Hoc LSD indicated that MDA on K-2 is significantly higher than K-1,  $p = 0.001$ . Compared to K-3, K-4, and K-5, MDA levels on K-2 is significantly higher,  $p = 0.001$  while MDA level on K-5 and K-1 have no significant difference,  $p = 0.421$ . (Figure 1. Sperm Quality (A.))

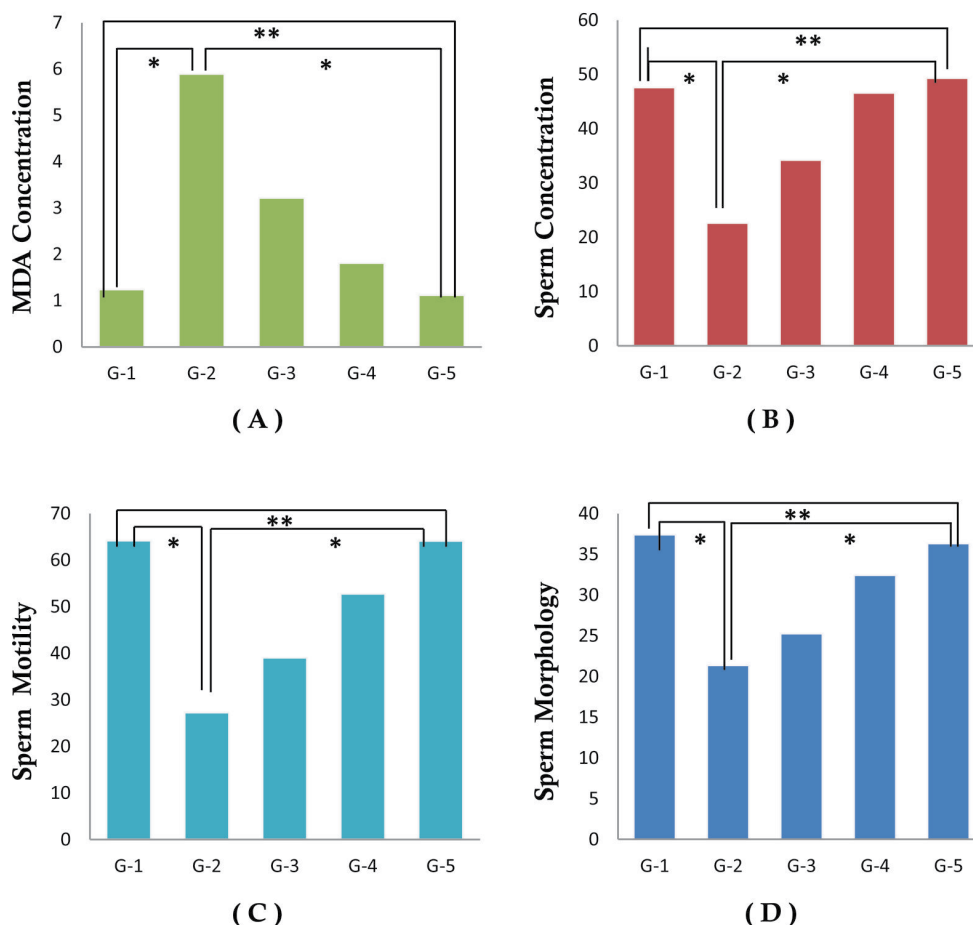
### Sperm Quality

#### 2. Spermatozoa Concentration

The highest spermatozoa concentration's mean is in group K-5 ( $49.32 \pm 1.06$ ), followed by group K-1

Table 1. Results of Mean Sperm Quality and MDA Levels

Variable	Groups					<i>p</i> ANOVA
	K-1 (n=6) Mean $\pm$ SD	K-2 (n=6) Mean $\pm$ SD	K-3 (n=6) Mean $\pm$ SD	K-4 (n=6) Mean $\pm$ SD	K-5 (n=6) Mean $\pm$ SD	
Amount (million /mL)	$47,60 \pm 1,56$	$22,62 \pm 1,55$	$34,25 \pm 1,48$	$46,60 \pm 2,42$	$49,32 \pm 1,06$	0,001
Morphology (%)	$37,43 \pm 1,42$	$21,39 \pm 1,77$	$25,26 \pm 0,56$	$32,45 \pm 2,46$	$36,34 \pm 0,90$	0,001
Motility (%)	$64,17 \pm 1,14$	$27,27 \pm 1,93$	$39,08 \pm 0,73$	$52,80 \pm 2,76$	$64,15 \pm 0,16$	0,001
MDA (nmol/ml)	$1,24 \pm 0,13$	$5,89 \pm 0,50$	$3,22 \pm 0,20$	$1,81 \pm 0,10$	$1,12 \pm 0,10$	0,001



\*\*  $P > 0.05$

\*  $P = 0.01$

Figure 1. Sperm Quality

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( $47.60 \pm 1.56$ ), K-4 ( $46.60 \pm 2.42$ ), K-3 ( $34.25 \pm 1.48$ ), and the lowest is in group K-2 ( $22.62 \pm 1.55$ ), (table 1). The ANOVA analysis indicated that average spermatozoa between groups show significant difference of,  $p = p < 0.05$ . The result of Post hoc LSD indicated that the average concentration of spermatozoa on K-5 is significantly higher compared to K-2,  $p = 0.001$ . Compared with K-3, K-4 and K-1, the average concentration of spermatozoa on K-5 is significantly higher,  $p = 0.001$  while the average numbers of spermatozoa on K-5 compared to K-1 have no significant difference,  $p = 0.086$ . (*Figure 1. Sperm Quality (B.)*)

### 3. Spermatozoa Motility

The highest spermatozoa motility's mean is in group K-1 ( $64.17 \pm 1.14$ ), followed by group K-5 ( $64.15 \pm 0.16$ ), kelompok K-4 ( $52.80 \pm 2.76$ ), K-3 ( $39.08 \pm 0.73$ ), and the lowest is in group K-2 ( $27.27 \pm 1.93$ ), (Table 1). The results of ANOVA analysis indicated that the spermatozoa motility mean between groups showed significant difference of  $p = p < 0.05$ . The Post hoc LSD indicated that the average of spermatozoa motility on K-1 are significantly higher compared to K-2,  $p = 0.001$ . Compared with K-3 and K-4, the spermatozoa motility mean on K-1 are significantly higher,  $p = 0.001$  while the average of spermatozoa motility on K-5 compared to K-1 are not significantly different,  $p = 0.981$  (*Figure 1. Sperm Quality (C.)*)

### 4. Spermatozoa Morphology

The highest normal spermatozoa morphology's mean found is in group K-1 ( $37.43 \pm 1.42$ ), followed by group K-5 ( $36.34 \pm 0.90$ ), group K-4 ( $32.45 \pm 2.46$ ), K-3 ( $25.26 \pm 0.56$ ), and the lowest is in group K-2 ( $21.39 \pm 1.77$ ), (Table 1). The ANOVA analysis indicated that the morphology of normal spermatozoa between groups indicated significant difference of,  $p = p < 0.05$ . The result of Post hoc LSD test indicated that the average of normal spermatozoa morphology on K-1 are significantly higher compared to K-2,  $p = 0.001$ . Compared to K-3 and K-4 the average of normal spermatozoa on K-1 are significantly higher,  $p = 0.001$  while the average of normal spermatozoa morphology on K-5 compared to K-1 have no significant difference,  $p = 0.241$ . (*Figure 1. Sperm Quality (D.)*)

## DISCUSSION

The result of this research indicated that cigarettes smoke exposure for 4 hours/ day for 21 days is proven can increase the MDA levels. This result is presented on the significant increase on MDA levels on negative control group compared to normal. This research is

supported by previous researches that smoking can increase ROS level and lower the antioxidant in semen (Saleh RA, *et al.*, 2003), which is followed by DNA fragmentation in spermatozoa (Zavos PM, *et al.*, 1998). On the other side, cigarette smoke exposure is proven to decrease spermatozoa quality. The quality of spermatozoa include concentration, motility, and morphology are significantly decrease on negative control group compare to normal. The decrease of spermatozoa quality was due to oxidative stress caused by the increase of free radicals induced by various chemical substances in cigarettes smokes such as nicotine, tars, and carbondioxide (Aina N, 2005, Karim D, 2011, Quaratul'ainy S, 2006, Tunc O, 2009). The increase of free radicals induced by cigarettes smokes are proven to cause peroxidation on double unsaturated acid from cell membrane to form MDA and cause oxidative stress during smoking. The increase of lipid membrane peroxidation and MDA formation will damage spermatogenic membran cell, disrupting transports of various ions important for proliferation and the growth of spermatogenic cell, disrupting DNA spermatozoa and increase spermatozoa apoptosis (Sukmaningsih A, 2009, Karim D, 2011). Furthermore, the increase of free radicals caused by cigarette smokes can also damage mitochondria membrane so the mitochondria will losts its potential membrane and disrupting mitochondria DNA and triggered cell apoptosis which effects on numbers, motility and spermatozoa morphology during spermatogenesis process (Faranita OV, 2009).

Reflecting on the result of MDA and spermatozoa quality on negative control group, furthermore we can deduct that before propolis administration, the MDA levels and spermatozoa quality on propolis group dose 2.9 mg, 5.4 mg, and 8.3 mg also experienced the increase of MDA levels and decrease of spermatozoa quality. Therefore, the propolis administration as antioxidant was expected to lower MDA levels and improve the quality of spermatozoa. The result of this research indicated that the propolis administration with doses 2.9 mg/200 gram bodyweight, 5.4 mg/200 gram bodyweight, dan 8.3 mg/200 gram bodyweight are proven to lower MDA level significantly compared to negative control group as well as the increase quality of spermatozoa which include concentration, motility and morphology. In fact, the administration of propolis with dose 8.3 mg/200 gram body weight is able to increase MDA levels and improving the quality of spermatozoa equal to normal. The result of this study is aligned with the research reported by Kardi 2015, Gluthathion antioxidant on adult male mice exposed with cigarette smoke can increase spermatozoa progressive motility. Apart

from that, some research also indicated that propolis antioxidants are proven to prevent the numbers decrease of spermatogonium cells, primary spermatocytes, and spermatid on rats (*Rattus norvegicus*) given physical stress (Putri Anggraeni Firdausyah, 2009).

Spermatozoa quality is affected by various spermatogenesis process in the testis include the levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) produced by anterior hypofisis, and testosterone produced by testicles Leydig cell. It is compulsory to conduct further research whether propolis have effect on various hormones concentration due to the cigarette smoke exposures.

## CONCLUSION

The administration of propolis extract on male rats Wistar strain exposed to cigarette smokes is proven to lower the MDA level and increase spermatozoa quality which include concentration, motility and morphology.

## REFERENCES

- Aina N. Pengaruh paparan asap rokok terhadap spermatogenesis dan kualitas spermatozoa mencit (*Mus musculus* L.) galur Swiss [skripsi]. UNS. 2005.hlm.1-2, 60-1.
- Agarwal A, Saleh RA, Bedaiway MA. Role of reactive species in the pathophysiology of human reproduction Fertil Steril. *Journal Reproduction*. 2003;(79):839-43.
- Agarwal A, Virk G, Ong C, du plessis SS. Effect of Oxidative Strss on Male Reproduction. *World J Mens Health*. 2014;32:1-17.
- Agarwal A, Prabakaran SA. Oxidative stress and antioxidants in male infertility: a difficult balance. *Iranian Journal of Reproductive Medicine*. 2005;3:1-8
- Cedikova M, Miklikova M, Stachova L, *et all*. Effects of the Czech Propolis on Sperm Mitochondrial Function. *Evidence-Based Complementary And Alternative Medicine* 2014; 2014: 248768.
- Capucho C, Sette R Souza SR, Castro Monteiro PFC, *et al*. Green Brazilian propolis effects on sperm countand epididymis morphology and oxidative stress. *Food Chem Toxicol* 2012; 50: 3956-62.
- Farooqui, T; Farooqui, A (2010) Molecular Mechanism Underlying the Therapeutic Activities of Propolis: A Critical Review. *Curr Nutr Food Sci* 6: 188-199.
- Faranita OV. Kualitas spermatozoa pada tikus wistar jantan diabetes melitus [skripsi]. UNDIP. 2009. hlm. 16-31.
- Gheldof, N., Wang, X.H., Engeseth, N.J. 2002. Identification and quantification of antioxidant components of honeys from various floral sources. *J Agric Food, Chem* 50:5870-7.
- Gocer H, Gulcin I. Caffeic acid phenethyl ester (CAPE): correlation of structure and antioxidant properties. *International Journal of Food Sciences and Nutrition*, 2011; 62: 821-825.
- Hlavackova L, Janega P, Cerna A, Pechanova O, Andriantsitohaina R, Babal P. Red Wine Polyphenol Affect the collagen Compotition in the aorta after Oxidative Damage Induced by Chronic Administration of CCl4. *Physiol Res*. 58: 2009. P. 337 – 44.
- Karim D. Pengaruh paparan asap rokok elektrik terhadap motilitas, jumlah sperma dan kadar MDA testis mencit (*Mus musculus* L.) [tesis]. Medan: Universitas Sumatera Utara; 2011:1,60-1.
- Koyu A. Ozguner F, Yilmaz HR, Uz E, Cesur G, Ozelik N. The protective effect of caffeic acid phenethylester (CAPE) on oxidative stress in rat liver exposed to the 900 MHz electromagnetic field. *Toxicology and Industrial Health*. 25. 2009. P. 429 – 34.
- Kardi, Pemberian Glutathion pada Mencit jantan dewasa yang dipapar asap rokok dapat meningkatkan Motilitas progresif spermatozoa. Program studi Magister Fakultas Kedokteran Udayana Denpasar, 2015.
- Mot, A.C., Damian, G., Sarbu, C., Silaghi, D.R. 2009. Redox reactivity in propolis: direct detection of free radicals in basic medium and interaction with hemoglobin. Department of Chemistry and Chemical Engineering, 'Babes-Bolyai' University, Cluj-Napoca, Romania. *Journal Medicine Food*.14(6):267-74.
- Nakajima, Y., Tsuruma, K., Shimazawa, M., Mishima. S., Hara, H. 2009. Comparison of Bee Products Based on Assays of Antioxidant Capacities. Nagaragawa Research Center. Department of Biofunctional Evaluation, Molecular Pharmacology, Gifu Pharmaceutical University,

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- 5-6-1 Mitahora-higashi, Gifu 502-8585. Japan. Published online by *Journal BioMed Central Medicine*, 1472-6882/9:4.
- Nadeem F, Fahim A, Bugti S. Effects of cigarette smoking on male fertility. *Turk j Med Sci*. 2012;42:1400-5.
- Putri Anggraeni Firdausyah, 052010101015; 2009: 76 halaman; Fakultas Kedokteran Universitas Jember.
- Quaratul'ainy S. Pengaruh vitamin E terhadap jumlah spermatozoa mencit jantan starin balb/c yang diberi paparan asap rokok [skripsi]. 2006. hlm.1-6.
- Rahmatullah P. Pneumonitis dan penyakit paru lingkungan. Dalam: Sudoyo AW, Setiyohadi B, Alwi I, Simadibrata M, Setiati S. Buku ajar ilmu penyakit dalam III. Edisi kelima. *Interna Publishing*; 2009.hlm. 2285.
- Russo A, Troncoso N, Sanchez F, Garbarino JA, vanilla A. Propolis protects human spermatozoa from DNA damage caused by benzo(a)pyrene and exogenous reactive oxygen species. *Life Sci* 2006 Feb 23;78(13):1401.6.epub 2006 Feb 7.
- Sherwood L., Jakarta: EGC. *Fisiologi Manusia dari sel ke system*. 6 ed. Jakarta: EGC; 2011.
- Sukmaningsih A. Penurunan jumlah spermatosit pakiten dan spermatid tubulus seminiferus testis mencit (*Mus musculus*) yang dipaparkan asap rokok. *Jurnal Biologi*. 2009;12:31-2.
- Subash P, Gurumunthy P, Sarasabharathi A, Cherian KM. Urinary 8-OHdG : A Marker of Oxidative Stres to DNA and Total Antioksidant Status in Essential Hypertension with South Indian population. *Indian Journal of Clinical Biochemistry*. 2010;25:127-32.
- Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, *et al*. Negative effect of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003; 79(3); 1597-1605.
- Somwanshi SD, Madole MB, Bikkad MD, Bhavthakar SS, Ajay G, Bhgwat S. Effect of cigarette smoking on Sperm Count and sperm Motility. *Journal of Medical education & Research*.2012;1:30-8.
- Tremellen K. Oxidative stress and male infertility, a clinical perspective. *Human reproduction update* 2008; 14: 243 – 58.
- Tunc O. Improvement in sperm DNA quality using an oral antioxidant therapy. *RBM online* 2009; 18 (6): 761 – 768.
- Zhang ZH, Zhu HB, Li LL, Yu Y, Zhang HG, Liu RZ. Decline of semen quality and increase of leukocytes with cigarette smoking in infertile men. *Iran J Reprod Med*. 2013;11:589-96.
- Zavos PM, Correa JR, Karagounis CS, Ahparaki A, Phoroglou C, Hicks CL, *et al*. An electron microscope study of the axonemal ultrastructure in human spermatozoa fram male smokers and nonsmokers. *Fertil Steril* 1998; 69: 430-434